Effect of Pupal Holding Density on Emergence Rate, Flight Ability, and Yield of Sterile Male Mediterranean Fruit Flies (Diptera: Tephritidae)

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Abstract. The Sterile Insect Technique (SIT) is commonly used to suppress or eradicate infestations of the Mediterranean fruit fly (or medfly) *Ceratitis capitata* (Wiedemann). Flies are mass-produced, sterilized, and shipped as pupae from the production facility to an eclosion and release facility. Pupae, and then emerging adults, are stored in eclosion towers consisting of 40–60 horizontally stacked, screen-paneled trays. In California, each tray is stocked with 350 ml of pupae, but this amount (the "pupal loading") varies among medfly SIT programs. Moreover, there exist no published reports regarding the potential impact of pupal loading on the performance of the adult sterile males. The goal of the present study was to compare two parameters—adult emergence and flight ability—across three pupal loadings, i.e., 250, 350, and 450 ml per tray. Two separate experiments were conducted at the eclosion-release facility in Los Alamitos, CA, which receives sterilized pupae from both Guatemala (Gflies) and Hawaii (Hflies). Results from both experiments revealed a negative impact of pupal loading level on flight ability, with a greater decline noted for Gflies than Hflies. Emergence rate was not affected markedly. The number of fliers produced per tower increased with pupal loading level of the constituent trays, but importantly the proportion of pupae that produced flight-capable was significantly lower for the 450 ml pupal loading level than the 250 or 350 ml pupal loading levels. Implications of these results for medfly SIT programs are discussed.

The Sterile Insect Technique (SIT) is widely used to suppress or eradicate infestations of the Mediterranean fruit fly (medfly), *Ceratitis capitata* (Wiedemann) (Enkerlin 2005). The SIT involves the production, sterilization, and release of large numbers of *C. capitata* males into the environment. Matings between sterile males and wild females result in the oviposition of infertile eggs, thus causing a decline in the wild population. The California Department of Food and Agriculture (CDFA) and the United States Department of Agriculture (USDA) operate a Preventive Release Program (PRP) in Southern California, with sterile medflies shipped as pupae from production facilities in Hawaii and Guatemala to an eclosion-release facility in Los Alamitos, CA.

This program, as well as those in Florida and Guatemala, employs a tower eclosion system. A tower consists of interlocking, screen-paneled, aluminum trays (76 x 76 x 2.5 cm, l:w:h) stacked on a wheeled base. Pupae are placed in a trough around the edge of a tray, and food (a sugar-agar gelatin) is placed on the central screen panel. Upon emergence, the flies move to

the screen and feed, and the puparia are left behind in the trough. The different programs use different amounts of pupae per tower tray: the California program uses 350 ml of pupae per tray, whereas the Florida and Guatemala programs use 400 ml of pupae per tray (D. Dean and P. Rendon, personal communication).

There exist no published reports examining the potential impact of pupal loading on the performance of the sterile males, and there is no accepted international recommendation for the appropriate loading level. The goal of the present study was to compare two parameters—adult emergence and flight ability—across three pupal loadings, i.e., 250, 350, and 450 ml per tray. Additionally, we estimated yield, on a per tower basis, as (i) the number of fliers produced and (ii) the ratio of the number of fliers produced to the number of pupae used.

Materials and Methods

Study insects and handling procedures. Andress et al. (2012) give a detailed description of the procedures used in handling pupae and adults, and here we provide an abbreviated account. All work was conducted during 2012–2013 at the David Rumsey Eclosion and Release Facility, Los Alamitos, California. Pupae are obtained from two rearing facilities, the CDFA Fruit Fly Rearing Facility, Waimanalo, Hawaii, and the USDA Medfly Rearing Facility, El Pino, Guatemala. Using similar protocols, these facilities produce flies of the same genetic sexing strain (Vienna 7/Tol-99), which, following heat-induced death of female eggs, allows production of males exclusively (Franz et al. 1996).

Upon arrival at the eclosion facility, the pupae are loaded into eclosion towers, which involves the tray-by-tray placement of pupae (350 ml pupae per tray) and adult food to build a complete tower (the day of pupal placement is termed Day 1). The loaded towers are housed inside climate controlled rooms until knockdown (chilling) and release of the eclosed flies. Peak emergence of adult flies occurs 2 d after pupae are placed in the towers (i.e., Day 3), and release occurs 2 d after peak emergence (i.e., Day 5). On the day of release, the towers are wheeled into a refrigerated trailer, where they are chilled for 45-80 min to immobilize the flies. Flies are then taken to aircraft for release.

Experiment 1: Tower trays as experimental units. On each of 15 dates, a single tower was set up that contained both Hawaii- and Guatemala-derived pupae (hereafter referred to as Hflies and Gflies, respectively). The tower contained 56 trays in total, with 18 test trays (3 loadings, 6 replicate trays per loading) set up for Hflies and Gflies, respectively, and 20 nontest trays (each with 350 ml of pupae). The test trays occupied designated, mid-level positions in the tower, and their positions were alternated across dates to ensure that Hflies and Gflies were held at similar heights over the entire test. Temperature and relative humidity were measured at 10-min intervals at four heights in the tower using Embedded Data Systems ibuttons (model DS1923 Hygrochron). These environmental parameters were uniform within and between towers and hence were excluded from the statistical analyses presented below. Over all sampling heights and towers, mean temperature varied only between 24.7–25.0°C, and mean humidity ranged only between 65.3% and 67.8%.

On the release date, the towers were moved into a chilled trailer for knockdown. We estimated emergence rate for the individual trays by collecting a 'teaspoon-full' sample of pupal casings from the trough and scoring the numbers of emerged, unemerged, or partially emerged flies for the first 100 pupal casings sorted and examined. In addition, 40

ml of flies were collected from each test tray. From each of these samples, 100 randomly selected flies were counted out and measured for flight ability following internationally accepted protocol (FAO/IAEA/USDA 2014), which briefly involved placing flies at the base of an opaque, vertical tube (whose interior surface was coated with talc to prevent escape by walking), counting the number of flies remaining in the tube after 2 h, and subtracting this number from 100 to determine the number of males capable of flight (i.e., fliers).

Both data for emergence rate and flight ability were analyzed using Restricted Maximum Likelihood (REML) to fit a standard least squares model. Initially, separate analyses were performed for Hflies and Gflies, respectively, with shipment date as a random effect and pupal loading and tray position in the tower as fixed effects. These initial analyses showed that tray position had no significant effect on emergence or flight ability for Hflies or Gflies. Accordingly, a second analysis using pooled data from the two sources included only pupal loading and source as fixed effects; shipment date was again treated as a random effect.

Experiment 2: Whole towers as experimental units. Data in this experiment were gathered only for Hflies. On each of 11 dates, nine towers were set up with three towers allocated to each of the three pupal loadings. All towers contained 52 trays. Over the 11 test dates, the towers were placed in the same nine locations in the same room with pupal loadings assigned randomly to the locations on each date. On six dates, an ibutton was placed within each tower and recorded temperature and relative humidity as noted above. As above, little variation was observed in temperature or humidity. Over all dates and towers, the average temperature and relative humidity within the towers were 24.5–25.0°C and 66%–70%, respectively.

After the towers were chilled, a teaspoon-full of pupal casings and 40 ml of adult flies were collected as described above from the 10th tray from the top for each of the towers. Estimates of emergence rate and flight ability were obtained following the procedures described above, and the data were analyzed using REML, with shipment date as a random effect and pupal loading and location within the holding room as fixed effects

Yield. We estimated yield, on a per tower basis, as i) the number of fliers produced and ii) the ratio of the number of fliers produced to the number of pupae used. For the sample trays (i.e., 10th from the tower top), pupal number was estimated by dividing the total weight of the pupal load by the weight of an individual pupa (using the average weight of samples of 150–200 individuals). This value was then multiplied by 52 trays to obtain an estimate of the total number of pupae placed in individual towers. For a given tower, the total number of pupae was then multiplied by observed emergence rate for the sampled tray, and this value (estimated number of emerged adults) was multiplied by the observed flight ability for the sampled tray to estimate the total number of fliers produced per tower. Yield estimates were analyzed using REML, with shipment date as a random effect and pupal loading as the single fixed effect (room location was not included, since [as shown below] it had no significant effect on emergence or flight ability).

Results

Experiment 1: Tower trays as experimental units. *Emergence rate*. Separate analyses for the two sources revealed differing effects of pupal loading on emergence rate. For each source, shipping date accounted for a large portion of the total variability in emergence (49.2% and 25.2% for Hflies and Gflies, respectively).

Among the fixed effects, tray position had no significant effect on emergence for either source ($P > 0.05$ in both cases), while increased pupal loading resulted in a significant decline ($F = 11.3$, $P < 0.001$) in the emergence of Gflies but had no detectable effect ($F = 0.001$, $P = 0.97$) on emergence of Hflies (Fig. 1). Despite the statistical significance detected for the Gflies, the absolute decrease in emergence was minor, from 92.7% at 250 ml to 90.4% at 450 ml. Emergence rates for Hflies were essentially constant (79.1% - 79.9%) across pupal loadings and consistently lower than rates observed for the Guatemala flies.

Analysis using data pooled over the two sources reflected the aforementioned trends: (i) shipment date accounted for a large proportion (22.4%) of the total variation, (ii) source had a significant effect ($P < 0.0001$), and (iii) pupal loading had a marginally significant ($P = 0.07$) effect, reflecting the differing results observed for this variable between Hflies and Gflies.

Flight ability. Separate analyses for the two sources revealed similar trends regarding flight ability. For each source, shipping date accounted for a large portion of the total variability in flight ability (54.8% and 60.8% for Hflies and Gflies, respectively). Likewise, once shipping date was taken into account, pupal loading had a significant effect on flight ability for flies from both sources ($F = 12.7$, $P < 0.001$ for Hawaii and $F = 106.8$, $P < 0.001$ for Guatemala), while tray position had no significant effect for flies from either source (Hawaii: $F = 1.2$, $P = 0.32$; Guatemala: $F = 1.6$, $P = 0.17$).

Based on these findings, analysis of the data pooled from both Hawaii and Guatemala included shipping date as a random factor and pupal loading and source as fixed effects. As before, shipping date accounted for a major part of the total variation in flight ability (50.4%), and both pupal loading ($F = 83.4$, $P < 0.001$) and source ($F = 453.6$, $P < 0.0001$) had highly significant effects on flight ability ($P < 0.001$ in both cases). On average, flight ability decreased with increasing pupal loading for flies from both sources, but the decline was more pronounced for Gflies than Hflies (Fig. 1). For Gflies, a 21% decline in mean flight ability was observed between the maximum and minimum pupal loadings (47.8%/60.4% = 0.79), whereas the corresponding decline for Hflies was only 5% (67.7%/71.3% = 0.95). At any given pupal loading, flight ability was lower for flies from Guatemala than those from Hawaii.

Experiment 2: Whole towers as experimental units. *Emergence rate*. Shipment date accounted for 35% of the variation observed in emergence rate, and neither fixed effect – pupal loading ($F = 2.6$, $P = 0.11$) nor room location ($F = 1.1$, $P = 0.40$) – had a significant effect on emergence. For data pooled over all samples ($n = 33$ per pupal loading level), average emergence rates were 76.1 ± 1.1%, 77.4 ± 1.0%, and 76.3 ± 1.0% for the 250, 350, and 450 ml pupal loadings, respectively.

Flight ability. Shipment date accounted for 17% of the variation observed in flight ability, and for the remaining variation pupal loading was found to have a significant impact on flight ability ($F = 17.8$, $P < 0.001$), whereas tower room location did not ($F = 0.9$, $P = 0.48$). Over all test dates, average flight abilities were: 250 ml pupae, 77.6 ± 1.1%; 350 ml pupae, 77.0% ± 1.0%; and 450 ml pupae, 71.4 ± 1.3%.

Yield. The total numbers of pupae placed in towers of 52 trays were estimated as 774,423 ± 8,527 for 250 ml pupae/tray, 996,353 ± 8,711 for 350 ml pupae/tray, and 1,278,360 ± 9,155 for 450 ml pupae/tray ($N = 33$ per pupal loading level). The estimated number of fliers produced per tower increased with pupal loading level (Fig.2); shipment date accounted for 19% of the variation, and pupal loading had

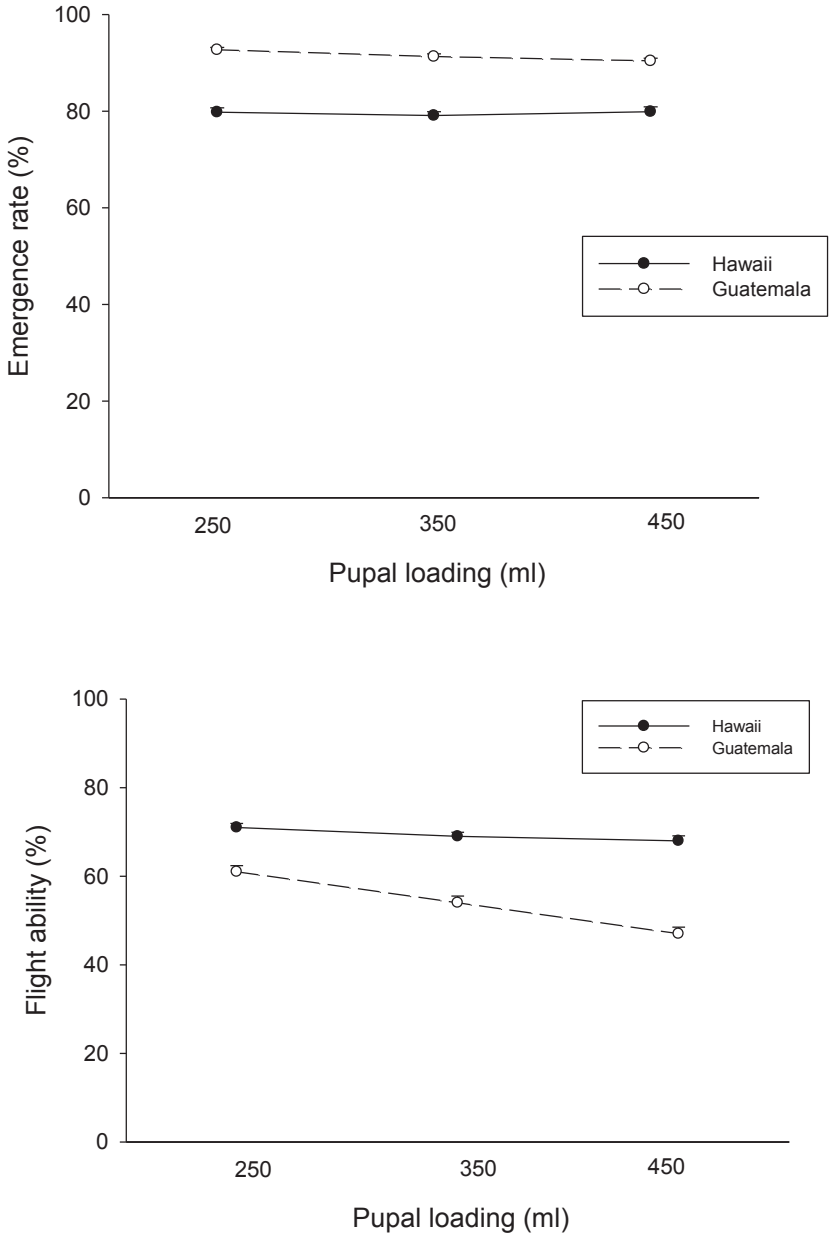

Figure 1. Emergence rate (top) and flight ability (bottom) for Hawaii- and Guatemala-derived flies when held at loadings of 250, 350, or 450 ml per eclosion tower tray. Bar heights represent mean values over all trays for a given density (15 dates X 6 trays per loading amount per date = 90 trays total), and error bars represent 1 SE.

a significant effect on the number of fliers produced ($F = 136.0$, $P < 0.001$). The proportion of pupae that produced flight-capable males varied in a non-monotonic manner (Fig. 2). Shipment date accounted for 26% of the variation, and pupal loading had a significant effect on the ratio of fliers-to-pupae ($F = 7.9$, $P = 0.006$). Average ratios did not differ significantly between towers loaded at 250 or 350 ml pupae/tray (0.59 vs. 0.60, respectively), and both of these values were significantly greater than the average ratio (0.54) observed for towers with trays loaded at 450 ml pupae/tray (at $P = 0.05$ in all cases, Tukey test).

Discussion

The level of pupal loading per tray appeared to have little effect on emergence rate, and, in fact, had no detectable effect on emergence of Hflies in either Experiment 1 or 2. A significant effect was noted for Gflies, but even then the decline in emergence rate was slight, from 92.7% at 250 ml to 90.4% at 450 ml. Pupal loading (and hence the degree of adult crowding) had a more marked effect on the flight ability of the adult males. Flight ability declined significantly with increasing pupae per tray for both Hflies and Gflies in Experiment 1 and for Hflies in Experiment 2. The magnitude of this effect differed substantially between flies from Hawaii and Guatemala. For the Gflies, flight ability decreased 21% from 250 ml to 450 ml pupae per tray compared to only a 5% decrease for Hflies.

In previous studies on the effect of pre-release chilling on flight ability (under standard pupal loading level of 350 m per tray), Andress et al. (2012, 2013a) found that the procedure of holding flies in towers itself, independent of chilling, suppressed the flight ability of Gflies more than Hflies. This earlier finding coupled with the present study indicate that tower storage has a greater negative impact on the flight ability of Gflies and that the difference between sources is more evident with increasing holding density per tower tray. A key factor may be the difference in the timing of emergence between Gflies and Hflies. Pupae from Guatemala are shipped when slightly older than pupae from Hawaii, and adults emerge approximately 12 h sooner from the Guatemala pupae than the Hawaii pupae. Thus, adults derived from Guatemala were subject to tower conditions (including crowding) for a longer interval than adults derived from Hawaii, which may have resulted in their lower flight performance.

Experiment 2, with its use of a single pupal loading density for all trays in a tower, allowed direct estimation of adult yield under different holding densities. Not surprisingly, the total number of fliers produced per tower increased with pupal loading per tray. In contrast, the ratio of fliers-to-pupae for towers whose trays were each loaded with 450 ml of pupae was significantly lower than ratios computed for towers having 250 or 350 ml of pupae per tray. These trends suggest two different strategies regarding pupal loading. If the program's goal is to maximize the number of flight-capable sterile males released, then the 450 ml pupal loading level would be preferred. Such a situation might reflect, among other factors, the financial cost of pupae and the mating competitiveness of the sterile males. If, for example, obtaining pupae represents a minimal cost to the program, then maximizing the fliers-to-pupae ratio is not critical, and lower ratios might be accepted if release numbers are increased. Similarly, if sterile males have low mating ability, then maximizing the field overflooding ratio (sterile:wild males) may be necessary for effective control via the SIT. Conversely, if the program's goal is to maximize rearing efficiency, then the 350 ml pupal loading level would be

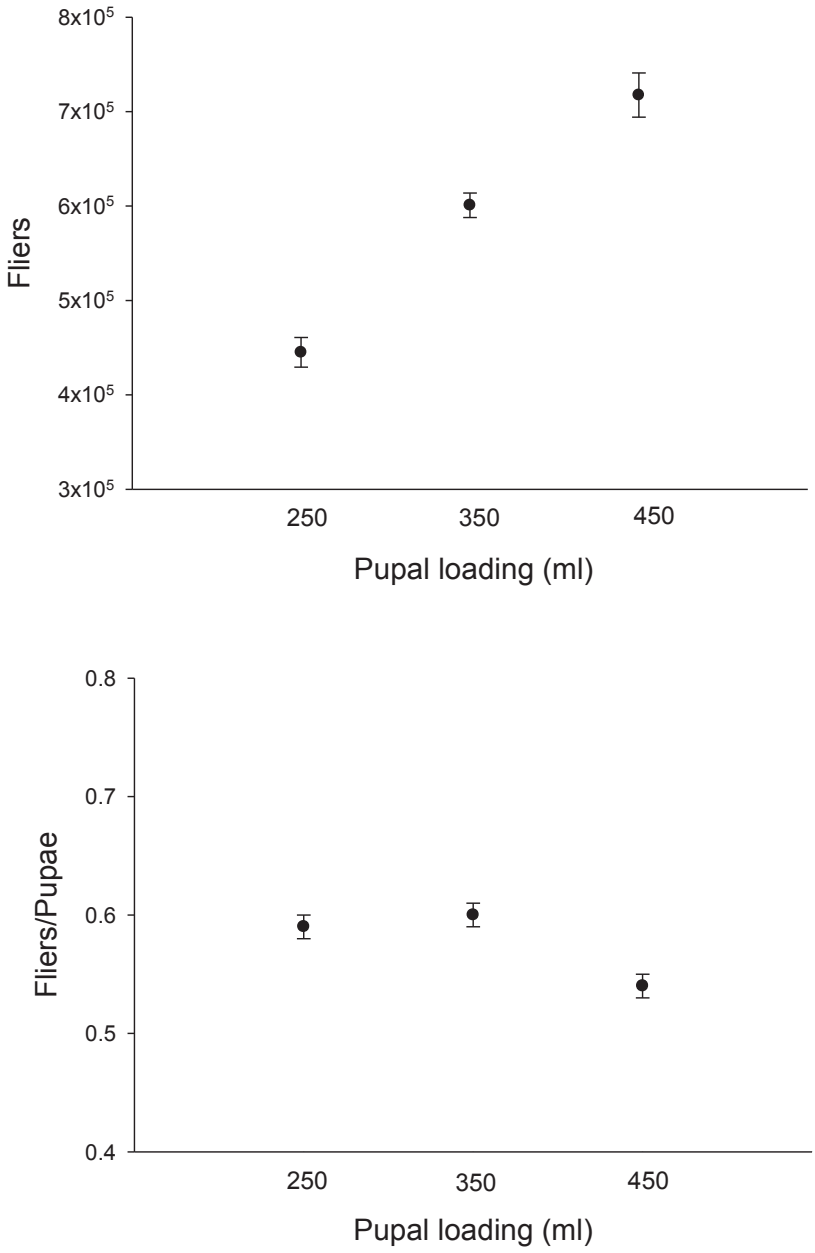

Figure 2. Estimated numbers of fliers produced per tower (top) and ratios of number of fliers produced to number of pupae placed per tower (bottom) in relation to the pupal loading level per constituent tray. Hawaii-derived flies were used exclusively; towers contained 52 trays. Symbols represent means over 33 towers per loading level (3 towers per test day x 11 test days); error bars represent ± 1 SE.

used (although having a similar fliers-to-pupae ratio, the 250 ml loading level produces fewer fliers and hence is less effective). Adopting the reverse argument used above, expensive pupae and sexually competitive sterile males might lead to a pupal loading strategy where efficient, and not necessarily maximal, release of sterile males is the operational goal.

We recognize that the two performance parameters measured here provide an incomplete assessment of the quality of sterile males and that the effect of pupal loading on mating competitiveness and dispersal should be examined as well. Unfortunately, non-trivial, logistic hurdles complicate measurement of these parameters: (i) the holding and eclosion of sterile male medflies occurs in a location (California) devoid of wild flies, thus precluding standard mating competitiveness trials and (ii) the low capture rate of aerially released flies (≈ 0.1%, Andress et al. 2013b) means that large numbers of flies (likely several million per pupal loading treatment per release) would be required for dispersal measurements, but such numbers are unavailable with the ongoing PRP. Alternate tests could be conducted (i.e., run mating trials with irradiated, wild flies and measure movement over short distances following ground release), but their significance would, unfortunately, remain questionable.

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